Attorney Docket No. 5405.239CT PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re: Williams
Serial No.: 10/625,134
Filed: July 23, 2003
For: *Use of polymorphism of the serotonin transporter gene promoter as a predictor of disease risk*

Group Art Unit: 1634
Examiner: J. Sitton
Confirmation No. 8271

Mail Stop Amendment
Commissioner for Patents
P.O. Box 1450
Alexandria, VA 22313-1450

Declaration of Redford Williams, M.D.
Pursuant to 37 C.F.R. § 1.132

I, Redford Williams, do hereby declare and say as follows:

1. I am a named inventor on US Provisional Application No. 60/162, 390 (the '390 provisional application) and US Application No. 10/625,134 (the '134 application) and of the subject matter claimed therein.

2. I have a Medical Degree (M.D.) from Yale University School of Medicine, New Haven, CT. I am a Professor of Psychiatry and Medicine at Duke University in Durham, North Carolina. I have been conducting research in the area of psychosomatic medicine for 40 years and have authored or co-authored more than 200 publications related to this area.

3. This Declaration sets forth data demonstrating that the association between the serotonin transporter gene promoter polymorphism (5HTTLPR) long allele genotype and larger mean arterial blood pressure rises in response to stress is significant across gender groups.

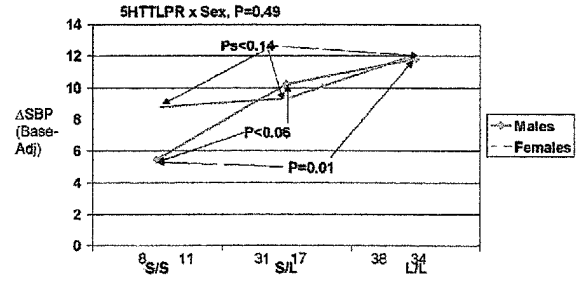
4. The figures below show data from the same sample of subjects described in the Williams et al. 2003 publication as described in the Declaration submitted January 5, 2007. Data that were presented in bar graphs in the previous Declaration are presented in the present Declaration as line graphs, with p-values. Data in these figures demonstrate in both males and females that carriers of the 5HTTLPR L allele (L/L or S/L) exhibited larger mean arterial blood pressure rises than those with the S/S genotype in response to the psychological stress protocols described in my patent applications and in Williams et al. (*Psychosom Med.* 63:300-305 (2001)).

In particular, the line graphs below show that, for systolic blood pressure (SBP), the 12 mmHg increase in females with L/L genotype is larger than that for females with both S/L and S/S, Ps<0.14. For males the S/L increase of 10.2 mmHg is larger than the 5.4 mmHg increase for S/S at P<0.06, and the 11.8 mmHg increase in males with L/L is larger than S/S at P=0.01.

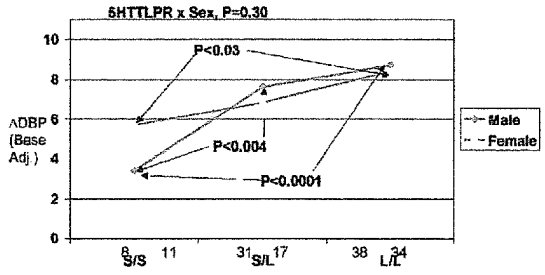
For diastolic blood pressure (DBP), the results are even stronger, with L/L females having larger DBP increase than S/S women at P<0.03; and males with S/S having smaller increase than S/L males at P<0.004 and L/L males at P<0.0001.

These results show that SBP and DBP rises during stress are larger in both males and females with L alleles. These data also show that for both SBP and DBP, the 5HTTLPR x Sex interactions are not significant -- P=0.49 for SBP and P=0.30 for DBP. This means that the effects of 5HTTLPR L allele on SBP and DBP increases in response to stress are not different in males and females -- i.e., are the same.

Systolic BP Response to Stress in Males and Females



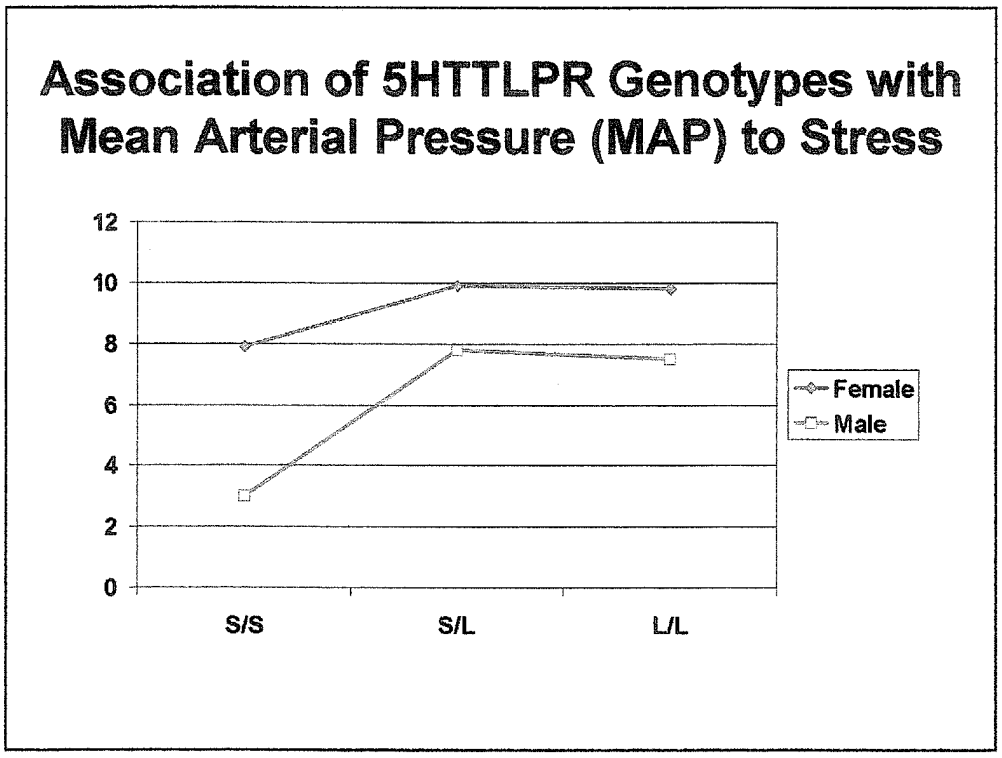
Diastolic BP Response to Stress in Males and Females



5. The figure below shows mean arterial pressure (MAP) data from the same sample of male and female subjects described above and as described in the Williams et al. manuscript that is currently In Press in *Psychosomatic Medicine* (copy appended hereto). Data in this figure demonstrate that both males and females that carry the 5HTTLPR L allele (L/L or S/L) exhibited larger mean arterial blood pressure rises than those with the S/S genotype in response to the psychological stress protocols described in my patent applications and in Williams et al. (*Psychosom Med.* 63:300-305 (2001); and *Psychosom Med* (2007), In Press.

As described in the enclosed manuscript (*Psychosomatic Medicine, In Press)* on page 14, "the increasing DBP reactivity as a function of the 5HTTLPR L allele was similar in men and women and in blacks and whites, with effects of 5HTTLPR genotype being significant in blacks, whites and males and at the trend level in females." As shown in the figure below, results for MAP are similar, with response to stress in males with S/S genotype significantly smaller than that in males with S/L (P=0.007) or L/L (P<0.01), while in females the MAP response of those with S/S genotype is smaller than that in females with S/L (P=0.26) or L/L (P=0.21), but the difference is not significant. However, as further explained in the manuscript on page 14, "Neither race nor sex interacted significantly with ..... 5HTTLPR genotypes to influence CVR [i.e., systolic and diastolic blood pressure and heart rate]." Additional analyses show that the

statistical interaction between 5HTTLPR genotypes and sex in predicting MAP response to stress is also not significant (P=0.49). This means that the two lines shown in the figure below representing MAP responses to stress in males and females are parallel – that is, they are not different from each other, and the effect of the 5HTTLPR L allele on MAP response to stress is the same in females as it is in males, even though the strength of this effect is significant in males and not in females. It also means that effects of 5HTTLPR genotypes on MAP response to stress can be tested in males and females combined, and when this is done, the significance level for the 5HTTLPR genotype effect is P=0.01.



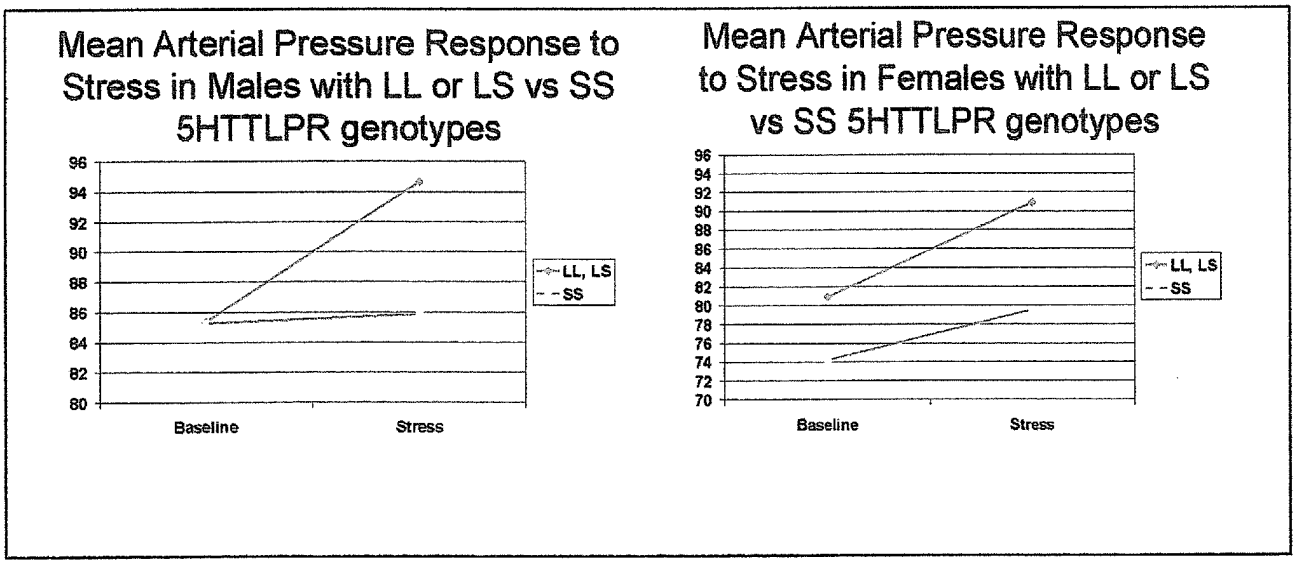
6. Figure 4 of the application shows that when males and females were considered together as a single group, those with 5HTTLPR SS genotype showed a nonsignificant MAP increase from baseline to stress, while those with LL or LS genotypes showed a highly significant MAP increase, and the 5HTTLPR x Period (baseline, stress) interaction was highly significant (P<0.0001), indicating that the MAP

response to stress was significantly different in L carriers than in those with SS genotype.

When these original MAP data are analyzed including sex in the analysis, the 5HTTLPR x Period interaction is still highly significant (P<0.005), but the 5HTTLPR x Period x Sex interaction is not significant (P=0.39), indicating that, as in results from the larger sample described above in Section 5, the larger MAP increase from baseline to stress is not different in males and females.

As shown in the figures below, using the original data on which Figure 4 of the application is based, the MAP increase from baseline to stress in those with SS genotype is not significant in males (P=0.79) or females (P=0.13). In contrast, among those with LL or LS genotype the MAP increase is highly significant, P<0.0001, for both males and females.

These results demonstrate that the larger MAP response to stress in L carriers that was found in the original data on which Figure 4 of the application is based was the same in males and females.



7. I hereby declare that all statements made herein of my own knowledge are true and that all statements made on information and belief are believed to be true; and further that these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code and that such willful false statements may jeopardize the validity of the application or any patent issued thereon.

Redford B. Williams — 11 October 2007

Redford Williams, M.D. — Date

Psychosomatic Medicine In Press, 28 September 2007

CHILDHOOD SOCIOECONOMIC STATUS AND SEROTONIN TRANSPORTER GENE POLYMORPHISM ENHANCE CARDIOVASCULAR REACTIVITY TO MENTAL STRESS

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Short title: Genetic and Environmental Effects on CV Reactivity

Word count: 7.351

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ABSTRACT

Objective: Cardiovascular reactivity (CVR) to mental stress has been proposed as one mechanism linking stress to pathogenesis of cardiovascular disease. The more transcriptionally efficient long (L) allele of a polymorphism of the serotonin transporter gene promoter (5HTTLPR) has been found associated with increased risk of myocardial infarction, and we found the long allele associated with larger CVR to mental stress in a preliminary study of 54 normal volunteers. We hypothesized that low socioeconomic status (SES) and the 5HTTLPR L allele are associated with increased CVR to stress in a larger sample and that SES and 5HTTLPR genotypes interact to enhance CVR to stress. **Methods:** Subjects were 165 normal community volunteers stratified for race, sex, and SES, who underwent mental stress testing. **Results:** Childhood SES as indexed by Father's Education level was associated with larger SBP ($P<0.05$) and DBP ($P=0.01$) responses to mental stress. The L allele was associated with larger SBP ($P=0.04$), DBP ($P<0.0001$), and HR ($P=0.04$) responses to mental stress compared to the short (S) allele. Subjects with the SS genotype and high Father's Education exhibited smaller SBP (5.2 mmHg) and DBP (2.9 mmHg) responses than subjects with LL genotype and low Father's Education (SBP, 13.3 mmHg, $P=0.002$; DBP, 9.7 mmHg, $P<0.0001$). **Conclusions:** Both the 5HTTLPR long allele and low SES, particularly during childhood, are associated with increased CVR to mental stress, which could account, at least in part, for the increased CVD risk associated with these characteristics. If confirmed in further research, these characteristics could be used to identify persons who might benefit from preventive interventions.

Key Words: blood pressure; cardiovascular diseases; genetics; heart rate; environmental stress

Abbreviations Used: CVD, cardiovascular disease; CVR, cardiovascular reactivity; 5HTTLPR, serotonin transporter promoter polymorphism; SBP, systolic blood pressure; DBP, diastolic blood pressure; HR, heart rate; SES, socioeconomic status; GCRC, General Clinical Research Center; CNS, central nervous system

Enhanced cardiovascular reactivity (CVR) to stress is one mechanism whereby psychosocial factors like hostility, depression, job strain, social isolation and low SES contribute to the development and course of cardiovascular disease (CVD). (1,2) Persons with larger blood pressure (BP) increases from resting levels during a broad range of mental tasks show more rapid progression of carotid atherosclerosis (3), especially among those of low SES (4) or who work in stressful jobs (5). Increased CVR to stress is also associated with increased stroke incidence in middle-aged men (6); increased development of coronary atherosclerosis in cynomolgus monkeys fed a high fat diet (7); increased insulin resistance (8); and increased blood lipid levels (9). In addition to predicting a higher incidence of hypertension (10), a larger BP response to a video game task predicted increased levels of a surrogate marker of atherosclerosis, coronary artery calcification, 13 years later in a large sample of healthy young black and white men and women (11). Prior research has identified environmental stress factors and candidate genes that are associated with both increased CVD risk and CVR as a potential pathogenic mechanism.

Low SES is an environmental factor that is clearly associated with increased risk of disease and death.(12,13) In particular, lower family SES during childhood is associated with increased mortality in adulthood, independently of adult SES, suggesting an enduring impact of adverse social and physical circumstances in childhood on adult health. (14) Mechanisms that could account for such an enduring effect include effects of early environment on psychosocial and behavioral risk factors (15) and increased insulin resistance (16) in adulthood. Lower SES is also associated with increased CVR to psychological stress among adolescents and young adults (17, 18), and a higher level of parental education buffers the effect of low neighborhood SES to increase CVR in healthy black adolescents.(19)

The role of genetic factors in CVR to stress is documented by twin studies finding moderate to strong heritabilities for systolic blood pressure (SBP), diastolic blood pressure (DBP), and heart rate (HR) responses to various laboratory stressors. (20-22) Molecular genetic studies have found associations between increased CVR to mental stress or adrenergic agonists and polymorphisms of several biologically plausible candidate genes, including those encoding for angiotensin converting enzyme (23), the angiotensin II type 1 receptor (24), and the beta-2 adrenergic receptor. (25-27)

There is also reason to consider the role in CVR of genes involved in the regulation of serotonin function. Serotonin receptors in the central nervous system (CNS) regulate effects of the sympathetic nervous system (SNS) on CV function, with the $5HT_{1A}$ receptor mediating decreased SNS outflow and the $5HT_2$ receptor mediating increased SNS outflow in animal models. (28) Treatment with selective serotonin reuptake inhibitors (SSRIs) is associated with decreased SNS outflow (29) and decreased CVD risk (30). In contrast, acute CNS serotonin depletion produces increased CVR to mental stress in recovered anxiety disorder patients. (31) It has been proposed that dysregulated CNS serotonergic function may enhance the expression of increased CVR to stress, hostility, and other psychosocial risk factors and biobehavioral mechanisms associated with increased CVD risk. (1)

A 44 base pair insertion/deletion polymorphism in the 5' regulatory region of the serotonin transporter (5HTT) gene, located on chromosome 17, has received considerable attention as a genetic variant with strong effects -- via effects on reuptake -- on serotonergic function. (32) The common variation in the 5HTT gene linked polymorphic region (5HTTLPR) consists of two alleles, with repeats designated "S" (short) and "L" (long). The S variant is

associated with decreased transcriptional efficiency and increased expression of neurotic traits in caucasian populations. (32)

We reported previously an association between the 5HTTLPR L allele and increased mean arterial blood pressure and HR reactivity to a set of laboratory stressors in a sample of 54 healthy volunteers (33), a finding that has been cited, along with serotonin-mediated changes in platelet functions, as one potential mechanism that might account for the observed association between the L allele and increased risk of myocardial infarction in three independent samples. (34-36) We have also found that 5HTTLPR genotypes are associated with cerebrospinal fluid (CSF) levels of 5-hydroxyindoleacetic acid (5HIAA), a brain endophenotype that reflects CNS serotonin turnover, but this effect varies as a function of both sex and race, with the S/S genotype being associated with higher CSF 5HIAA levels in women and blacks, but with lower levels in men and whites. (37) However, other studies have failed to demonstrate clear correlations between 5 HTTLPR genotype and CSF 5 HIAA (38) or serotonin transporter binding in brain. (39) Because these studies did not include African Americans (38) or test for race or gender moderation of 5HTTLPR effects (38,39), it must be concluded that the relationship between 5 HTTLPR and CNS serotonin function remains to be clearly defined. Rhesus monkeys with the less active s allele of a 5HTTLPR variant analogous to that found in humans have lower levels of CSF 5HIAA, but only if they have been exposed to the early adversity of being separated from the mother for the first six months of life and reared with peers. (40) This finding provides direct evidence of the importance of early adversity in affecting CNS serotonin function, as well as the moderation of such effects by 5HTTLPR genotype.

Guided by the findings reviewed above and observations that gene-environment (GxE) interactions can play an important role in human biological, cognitive and emotional responses

associated with acute and chronic exposures to current as well as previous stressors (41-43), we hypothesize that: a) low SES, especially during childhood, will be associated with increased CVR to mental stress; b) persons carrying the 5HTTLPR L allele will exhibit increased CVR to mental stress; and c) low SES and the presence of the L allele will interact to produce enhanced CVR to mental stress. We tested these hypotheses in a sample of healthy normal community volunteers that we have studied over the past five years.

Methods

Subjects were admitted to the General Clinical Research Center (GCRC) at Duke University Medical Center for a 2.5 day protocol that included lumbar puncture to obtain CSF (for assay of monoamine metabolites as reported elsewhere, 37), followed by randomization to either CNS serotonin enhancement (using tryptophan infusion) or CNS serotonin depletion (using tryptophan depletion) arms, with sham infusion or depletion on the first test day followed by active depletion or infusion on the second test day. Subjects also underwent mental stress testing at the point of expected maximal serotonin enhancement or depletion, with monitoring of cardiovascular function and collection of blood samples to assess neuroendocrine and immune system parameters. Since the focus of this report is on the effect of 5HTTLPR, SES, and their interaction on CVR to stress in the normal state, we report stress response data here only for the first day's testing in the sham depletion and infusion arms, when CNS serotonin function has not been manipulated. Results relating to effects of serotonin manipulations on responses to stress testing will be the subject of subsequent communications.

Subjects were recruited during the period 1998-2003 via advertisements in the public media, inclusion in the community newsletter sent with the county water bill, flyers posted throughout the community, via outreach screening events at civic organizations and other public

events, and in paid advertisements such as the back of supermarket tapes. This protocol required that subjects not be at risk due to the study procedures and that subject characteristics not hamper interpretation of the findings, making it important to ensure a sample population in good current health. Therefore, all subjects underwent a comprehensive examination using a modified SCID (by KMG) as well as medical history, physical exam, electrocardiogram, chest radiograph, hemoglobin, hematocrit, white cell count, and blood chemistries to rule out current psychiatric and medical disorders. Use of any prescription drugs, as well as use of illegal drugs (as detected by a urine screen prior to entry into study) were grounds for exclusion.

As part of the study design, participants were recruited according to their current education and household income levels in order to have approximately equal groups of low and high SES. Thus, two categories of income were used—below or equal to $24,900 and above $24,900, based on the 40th percentile rank of household incomes in Durham County according to the 1990 Census (44). The low SES category includes those who had income of less than or equal to $24,900 and who had less than a college degree. The high SES group included those who had income greater than $24, 900, regardless of education, or those with a college degree. As described in Burroughs et al. (44), the final sample consisted of 165 subjects with 98 high and 67 low SES, 91 male and 74 female, and 94 African American and 71 Caucasian (based on self-description) subjects, ages 18-50 (mean, 35.1). The stringent medical and psychiatric screening requirements made it harder for lower SES and women volunteers to qualify for the study. Included in this final sample are the 54 subjects in whom we found increased CVR among 5HTTLPR L carriers in our earlier, preliminary report. (33) With the addition of 111 new subjects, the final sample is more balanced in terms of SES (high vs low), sex and race and contains three times as many subjects with the SS genotype.

This study was approved by the Duke University Medical Center Institutional Review Board, and an IRB-approved form was used to obtain written informed consent from all subjects.

Procedures -- Subjects reported to the GCRC during the early afternoon. After completing admission procedures, and without a period of bed rest, lumbar puncture was performed by a board-certified anesthesiologist. Between 11AM and noon on the first test day, following the sham depletion or infusion, all subjects underwent a 45-minute mental stress protocol involving a 5-minute baseline rest period followed by oral reading from a neutral text, anger recall (oral report of a recent situation that made you angry), a second neutral text reading, and sadness recall (oral report of a recent situation that made you sad), with 5-minute rest periods between each 5-minute stress period. The reading and anger and sadness recall tasks were performed in the presence of two research assistants, thereby adding an element of public speaking stress.

Measures -- Genomic DNA was extracted by standard procedure (Puregene D-50K Isolation Kit, Gentra, Minneapolis, MN) from fresh or frozen samples of peripheral blood collected from the subjects. Polymerase chain reaction amplification to generate a 484- or 528-base pair fragment corresponding to the short (S) and long (L) 5HTTLPR alleles, respectively, was carried out as described elsewhere. (32) Consistent with other studies (45), allele frequencies varied significantly as a function of ethnicity, with African Americans having 72% L alleles and 28% S alleles, compared to 60% L and 40% S alleles in Caucasians ($X^2 = 4.74$, 1df, $P < 0.05$).

A Critikon Automatic Vital Signs Monitor was used to measure SBP, DBP, and HR at one-minute intervals during the Rest and Stress periods of the mental stress testing protocol.

Childhood SES was determined by participant's recall of parental education. Retrospective data on childhood SES has been shown to be valid in empirical studies. (46) Parent's education was classified as 'high' if they had above 12 years education and 'low' if they had 12 years of education or below. This served as a natural break point in level of education between high school and post-secondary education and provided approximately equal numbers of mothers and fathers with high vs. low education levels. The correlations between subjects' current SES and their parents' education levels were only modest, $rs>0.15$, $Ps>0.07$.

Statistical analyses -- Multiple regression in the form of Analysis of Covariance (ANCOVA) was used to evaluate baseline adjusted changes in cardiovascular measures between mean level across all rest and mean of all stress periods as a function of 5HTTLPR genotypes (LL, LS, SS) and SES (both current and childhood, using parents' education levels). We used mean SBP, DBP and HR levels across all rest and stress periods in these analyses, because these aggregated indices of CV function provide more reliable assessments. (47) The dependent variable was the delta from the mean of all rest periods to mean of the four stress periods, with the first pre-stress level as the covariate. To reduce the potential for false positive results due to population stratification, race was also included as a covariate in all analyses involving the total sample. To test for the possible effects of gene-environment interactions, categorical variables for SES and for gene phenotype, with their interaction, were modeled. When the interactions uniformly proved far from significant, the interactions were dropped from the models to more properly evaluate the main effects of SES and gene. This interaction deletion was done to avoid possible confounding of the main effects tests due to the imperfectly balanced design

Sample sizes in different analyses vary due to inadequate DNA extraction (N=1), missing data on mother's (N=8) or father's (N=20) education. Preliminary analyses showed that arm of

study (tryptophan depletion vs enhancement) had no impact on CVR to stress as a function of SES indices or 5HTTLPR genotypes on the first test day, when sham depletion or enhancement was used. In prior research on this sample we found that the association between 5HTTLPR genotypes and CSF 5HIAA is moderated by both race and sex, with the SS genotype having opposite effects on 5HIAA in men vs women and blacks vs whites. (37) However, preliminary analyses found no moderation of 5HTTLPR or SES effects on CVR by either sex or race; except for the father's education x race effect on SBP reactivity (P=0.11), all other interactions had Ps>0.37. Therefore, in all analyses we collapsed the sample across tryptophan arms (day 1) and both race and sex groups.

Results

We first evaluated associations between current and childhood SES indices and systolic blood pressure (SBP), diastolic blood pressure (DBP) and heart rate (HR) reactivity comparing the mean level during the rest periods with the level averaged across the four stress periods. There were no baseline differences as a function of any of the SES measures. As shown in Table 1, in models examining the joint effects of father's or mother's education and subject's current SES, there were no significant associations between current SES and CVR to the stress protocol. In contrast, childhood SES, as indexed by father's or mother's education level, showed significant (father's education) or trend (mother's education) associations with SBP and DBP changes during the stress protocol. There were no significant interactions between mother's or father's education and subject's SES in predicting CVR. Subjects with lower father's education levels exhibited SBP and DBP responses that were 25% larger than those with higher father's

education levels. These effects of father's education on CVR did not vary as a function of race (Ps, 0.11-0.73) or sex (Ps, 0.53-0.84) .

Table 1 About Here

Because father's education level was the most robust SES indicator associated with CVR to mental stress, we next evaluated joint effects of father's education and 5HTTLPR on CVR. Baseline SBP was lower, $F(2, 139) = 4.27$, $P=0.03$, in SS (104 +/- 2.8 [SE] mmHg) subjects than those with LS (111+/-1.7 mmHg; $P=0.03$) and LL (112+/-1.4 mmHg, $P=0.01$) genotypes. Baseline DBP was lower, $F(2, 139)=3.02$, $P=0.05$, in SS subjects (63+/-2.0mmHg) than those with LL (68+/-1.0 mmHg, $P<0.05$) but not LS (66+/-1.2 mmHG, $P=0.28$) genotypes. Baseline HR did not differ as a function of genotype. These effects of 5HTTLPR genotype on baseline SBP and DBP were similar in blacks and whites: mean SBP/DBP in blacks was 103/62 mmHg for those with SS, 111/65 mmHg for SL and 114/68 mmHg for LL; in whites the levels were 103/65 mmHg for SS, 111/66 mmHg for SL and 110/69 mmHg for LL. Further documenting that the effects of 5HTTLPR on baseline BPs were not due to the presence of more whites with the SS genotype in the sample (12 vs 7 among blacks), there were no race differences in baseline SBP (109 mmHg in blacks, 108 mmHg in whites, $P=0.73$) or DBP (64 mmHg in blacks, 67 mmHg in whites, $P=0.22$).

Race was entered and then, to control for these baseline differences, baseline levels of SBP, DBP and HR were entered along with father's education, subject's 5HTTLPR genotype and their interaction in models with changes from averaged rest periods in CV levels averaged across the four stress periods as the dependent variable. As shown in Table 2, independently of

5HTTLPR genotype, subjects with lower father's education showed larger SBP (32%) and DBP (39%) responses than those with higher father's education. Independently of father's education level and despite their higher resting SBP and DBP, subjects with the LL genotype showed larger SBP (58%, P=0.,02), DBP (100%, P<0.0001)) and HR (58%, P=0.01) responses than those with the SS genotype. LS subjects showed intermediate responses that were significantly different from both LL (P=0.04) and SS (P=0.003) subjects for DBP and different from SS (P=0.02) only and similar to LL for HR. Father's education by 5HTTLPR interactions were not significant for any CV response parameter. Models substituting mother's education or subject's SES for father's education produced similar patterns of results, but with less robust effects of these SES indicators on CVR when considered jointly with 5HTTLPR genotypes.

Table 2 About Here

The absence of significant SES x 5HTTLPR interactions using any SES index indicates that effects of both SES and 5HTTLPR genotype on CVR did not vary in a multiplicative way. The finding that both father's education level and 5HTTLPR genotype were independently associated with SBP and DBP reactivity indicates, however, the presence of a situation in which both the environmental exposure (low childhood SES as indexed by father's education) and the genotype (5HTTLPR LL genotype) can still have some form of joint effect on disease risk, but with the possibility that their combined effect on risk can be higher or lower than when they occur alone. (48) As shown in Figure 1, the combination of low father's education level and the 5HTTLPR LL genotype is associated with remarkably large *additive* effects on CVR. The 13.3 mmHg increase in SBP during stress in those with low father's education and the LL genotype is

significantly larger (Ps < 0.05) than all other groups and 2.6 times and 8.1 mmHg larger (P=0.002) than that seen in those with high father's education and the SS genotype. The low father's education/LL group's 9.9 mm Hg DBP response is larger (Ps <0.01) than all the other groups and 3.7 times and 7.2 mmHg larger (P<0.0001) than the DBP response in the high father's education/SS group. Also noteworthy is that, despite their lower baseline levels, the high father's education/SS group's SBP (Ps, 0.17-0.002) and DBP (Ps ≤0.05-0.0001) reactivity are smaller than all the other groups.

Figure 1 About Here

Neither race nor sex interacted significantly with SES indices or 5HTTLPR genotypes to influence CVR. As shown in Figure 2, the increasing DBP reactivity as a function of the 5HTTLPR L allele was similar in men and women and in blacks and whites, with effects of 5HTTLPR genotype being significant in blacks, whites and males and at the trend level in females.

Figure 2 About Here

Results obtained in analyses in which parental education level was modeled as a continuous variable did not differ in any material way from those reported above using the high/low dichotomy for parental education.

Discussion

These findings support our first two hypotheses. The finding of increased SBP and DBP reactivity in persons with low childhood SES as indexed by father's education is consistent with prior research (17-19) showing increased CVR as a function of low SES, particularly during

childhood. The association of the 5HTTLPR LL genotype with increased SBP, DBP and HR reactivity in a sample that is three times larger and more balanced with respect to race, sex and 5HTTLPR genotypes than in our preliminary report (33), especially the robustness of the association with DBP reactivity, suggests the presence of an acute, within-subjects GxE effect, such that the influence of genotype on CV function is larger during mental stress than during the rest periods. Considered together with evidence that increased CVR contributes, presumably via more frequent endothelial injury, to the development of CVD (3-11), the parallel between the increased CVR in persons carrying the L allele in this study and the association of the L allele with increased risk of myocardial infarction in three independent studies (34-36) suggests that increased CVR could be one mediator of the association between the L allele and CVD risk. Increased platelet serotonin uptake has also been observed in persons with the 5HTTLPR L allele (49), and could combine with increased CVR to speed atherogenesis and contribute to the precipitation of acute CHD events.

The lack of a significant statistical (i.e., multiplicative) interaction between SES indices and 5HTTLPR genotype indicates there is not a differential effect of this chronic environmental exposure on CVR as a function of genotype and that effect of genotype does not vary as a function of exposure – indicating that our third hypothesis was not supported by the findings. Nevertheless, the markedly larger SBP and DBP responses to the stress protocol in subjects with LL genotype *and* low father's education compared to those with SS genotype *and* high father's education (Figure 1) suggests that *additive* effects of environmental exposure and genotype can be quite large and likely to have clinically significant effects on risk. It has been reported, for example, that, independently of resting BP, there is a 24% increased odds of having detectable coronary calcium for each 10 mm Hg change in SBP during CVR testing 13 years earlier. (11)

The 8.1 mmHg difference in SBP reactivity between subjects with LL genotype and low father's education and those with SS genotype and high father's education suggests, therefore, that persons with the LL genotype and low father's education will exhibit a 19% higher incidence or prevalence of CVD compared to those with SS and high father's education. It will be possible to test this prediction in extant samples of healthy people or CVD patients with DNA available and among whom father's education levels are already known or can be readily determined. The higher baseline SBP and DBP we find in those carrying the L allele could add to the effect of the L allele on CVR to stress in potentiating the development of atherosclerosis.

The increased frequency of the 5HTTLPR L allele observed in African-derived populations (45) could be a contributor, via the associated increased CVR to stress, to the increased CVD incidence observed in African Americans, as well, for example, as the increased prevalence of hypertension among Zulus living in South African urban vs. rural settings. (50) If the L allele is playing such a role to increase stress effects on CVD risk in African-derived populations, the SS genotype should be more frequent, despite it's overall rarity (<10%), in those African Americans who are normotensive or free of other forms of CVD.

In contrast to moderation by both race and sex of the serotonin brain endophenotype indexed by CSF 5HIAA levels we reported previously (37), we find now in the same sample of subjects that the association of the L allele with increased CVR is similar in men and women, blacks and whites. We have no ready explanation for this difference between the race and sex moderation of the 5HTTLPR effect on whole brain serotonin turnover as reflected in 5HIAA levels, and the lack of race or sex moderation of CVR to stress. One potential explanation stems from the fact that 5HTTLPR genotype's influence on serotonergic regulation of sympathetic outflow could occur in the hypothalamus and brainstem, via effects on sympathoinhibitory

$5HT_{1A}$ and sympathostimulatory $5HT_2$ receptors (28). These brain regions are distant from the site of lumbar puncture, and serotonin turnover at these sites may not be be reflected in 5HIAA levels measured in CSF from lumbar puncture.

We recently found (51), in the same sample on which this report is based, that the association between 5HTTLPR genotype and depressive symptoms in persons whose father had a low education level does vary as a function of gender. In persons whose father had more than 12 years of education, there was no effect of 5HTTLPR genotype on levels of depressive symptoms. In those whose father had 12 or fewer years of education, however, higher levels of depressive symptoms were seen in men with the LL genotype, while in women it was the SS genotype that is associated with higher levels of depressive symptoms. Taken together with the findings reported here with respect to CVR to stress, these findings suggest that among men the LL genotype may be particularly harmful, because it is associated with both increased CVR to acute stress and increased depressive symptoms in the setting of chronic stress, as indexed by having a father with a low education level. Women with the LL genotype also show increased CVR to acute stress, but appear to be protected against the effect of chronic stress on depressive symptoms.

The present data do not permit us to establish the mechanism responsible for the larger CVR in those with the LL genotype. The effect could be mediated by the aforementioned effects of 5HTTLPR on CNS receptors that influence sympathetic outflow. Alternatively, or in addition to a CNS locus, the larger CVR in LL subjects could be due to effects on peripheral cardiac and/or vascular function. It has been shown, for example, that patients with chronic obstructive pulmonary disease who carry the LL genotype have significantly higher pulmonary artery pressure than those with LS or SS genotype. (52)

We are aware of only one other report (53) of effects of 5HTTLPR on CVR to mental stress. In that study the SS genotype interacted with sex to predict HR reactivity only (there were no effects on SBP and DBP reactivity) to the Stroop Color-Word Test and mental arithmetic in a sample of 131 Mz and 60 Dz twin pairs. In women, the SS genotype was associated with significantly larger HR reactivity, while in men there was a trend to decreased HR reactivity in those with SS genotype. Effects of SES on CVR were not reported. Other than differences between this study and ours in terms of the samples (twins vs a community sample) and stress tasks (cognitive vs. emotion-eliciting) we have no ready explanation for the similarity to our results for HR reactivity in men with an opposite effect (larger reactivity in SS) in women. It is possible that the cognitive tasks used in their study elicited primarily a cardiac response that was reflected mainly in HR, while the emotion-eliciting tasks in our current study also elicited a stronger vascular response, as reflected in the very robust effects of 5HTTLPR genotype on DBP reactivity. Ultimately, replication studies will be required to determine the effect of 5HTTLPR genotype on CVR to stress and, of equal or greater importance, the development of CVD. We are currently conducting research to address both these questions. In the meanwhile, the congruence between the reported increased platelet serotonin uptake in persons carrying the 5HTTLPR L allele (49), our finding of increased CVR to stress in those carrying the L allele and the finding of increased MI risk in those carrying the L allele in three independent studies (34-36) highlights the importance of continuing this line of research.

A limitation in the present study is the small number of subjects – 7 -- with both high father's education and the SS genotype, which could decrease confidence in the finding of very low CVR in this group. Reducing this concern, however, is the equally significantly higher CVR (see Figure 1) in the 40 subjects with low father's education and the LL genotype. Recent

research (54) has revealed the existence of a common single base substitution (A→G) within the 5HTTLPR L allele, with the rarer (10-15% in Caucasians, 24% in African Americans) L_G allele showing reduced transcriptional efficiency, comparable to that of the S allele, while the L_A allele is about twice as transcriptionally efficient as the S or L_G alleles. Rather than being responsible for our findings, however, the presence of the less functional L_G allele among the LL or LS subjects of our study would dilute the effects of the more active L_A allele, making it harder to find CVR differences between SS and LL subjects. The difference in CVR between LL and SS subjects in this study represents, therefore, a conservative estimate of the association of 5HTTLPR genotypes with CVR. Another potential limitation is the potential of the sham tryptophan depletion or infusion to have affected CVR on day 1 of our protocol. We consider this unlikely to account for our findings, however, because a preliminary analysis of our data that included study arm as a covariate showed that study arm did not affect the results.

If further research demonstrates the predicted increase in CVD prevalence or incidence among persons with the LL genotype and a low father's education, such persons could be targeted for behavioral and/or drug interventions with the potential to reduce CVR to stress. Beta-blockers would be one obvious pharmacologic approach to reduce CVR to stress. The use of selective serotonin reuptake inhibitors has been reported in an observational study to reduce risk of myocardial infarction (30), suggesting that these agents also deserve consideration as a means of reducing CVD risk associated with the L allele. There is also preliminary evidence from research in CHD patients that behavioral training in stress coping skills has the potential to reduce CVR to stress. (55) The stronger impact of childhood SES, as indexed by father's education level, on CVR suggests that such interventions could be more effective if instituted during childhood or adolescence.

In conclusion, our findings show that SBP, DBP and HR reactivity to a set of emotion-eliciting mental stressors are larger in a sample of healthy adults who are carriers of the 5HTTLPR L allele or whose childhood environment was of lower SES. Participants with the combination of the LL genotype and lower childhood SES showed marked elevations of CVR. These data support the hypothesis that increased CVR to mental stress could partially explain the increased CVD risk that has been associated with the L allele in three independent studies. If the combination of lower childhood SES and the LL genotype is found associated with increased coronary atherosclerosis and/or to predict the prevalence and incidence of clinical CVD in future research, it would indicate that persons carrying the LL genotype and whose childhood environment was of lower SES could be targeted for trials of interventions aimed at preventing the development of CVD.

Sources of Funding: National Heart, Lung, and Blood Institute grant P01HL36587 (RBW), Clinical Research Unit grant M01RR30, and the Duke University Behavioral Medicine Research Center. **Disclosures:** Redford Williams has a patent pending on use of 5HTTLPR L allele as a marker of increased CVD risk due to stress; Redford Williams is a founder and major stockholder in Williams LifeSkills, Inc.

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Table 1. Joint effects of parent's education (Father's Educ; Mother's Educ) and subject's current socioeconomic status (Subject's SES) on mean cardiovascular changes (baseline adjusted) across the four stress periods. Separate models were run testing father's education and subject's SES and mother's education and subject's SES.

SES Parameter(N)	SBP Change*	DBP Change	HR Change
Father's Educ			
Low (75)	11.7(0.8)	8.5(0.4)	8.6(0.5)
High (65)	9.5(0.9)	6.9(0.5)	7.8(0.6)
P	**0.05**	**0.01**	0.31
Subject's SES			
Low (85)	11.0(0.9)	8.1(0.5)	7.7(0.7)
High (55)	10.2(0.7)	7.3(0.4)	8.7(0.5)
P	0.50	0.23	0.25
Mother's Educ			
Low (73)	11.6(0.7)	8.3(0.4)	8.1(0.5)
High (79)	9.7(0.8)	7.3(0.5)	8.1(0.6)
P	0.09	0.11	0.95
Subject's SES			
Low (58)	11.2(0.9)	8.2(0.5)	7.5(0.6)
High (94)	10.2(0.7)	7.4(0.4)	8.7(0.5)
P	0.38	0.19	0.11

* Mean (S.E.)

Table 2. Effects of father's education level and 5HTTLPR genotypes on mean cardiovascular changes (adjusted for baseline and race) across the four stress periods.

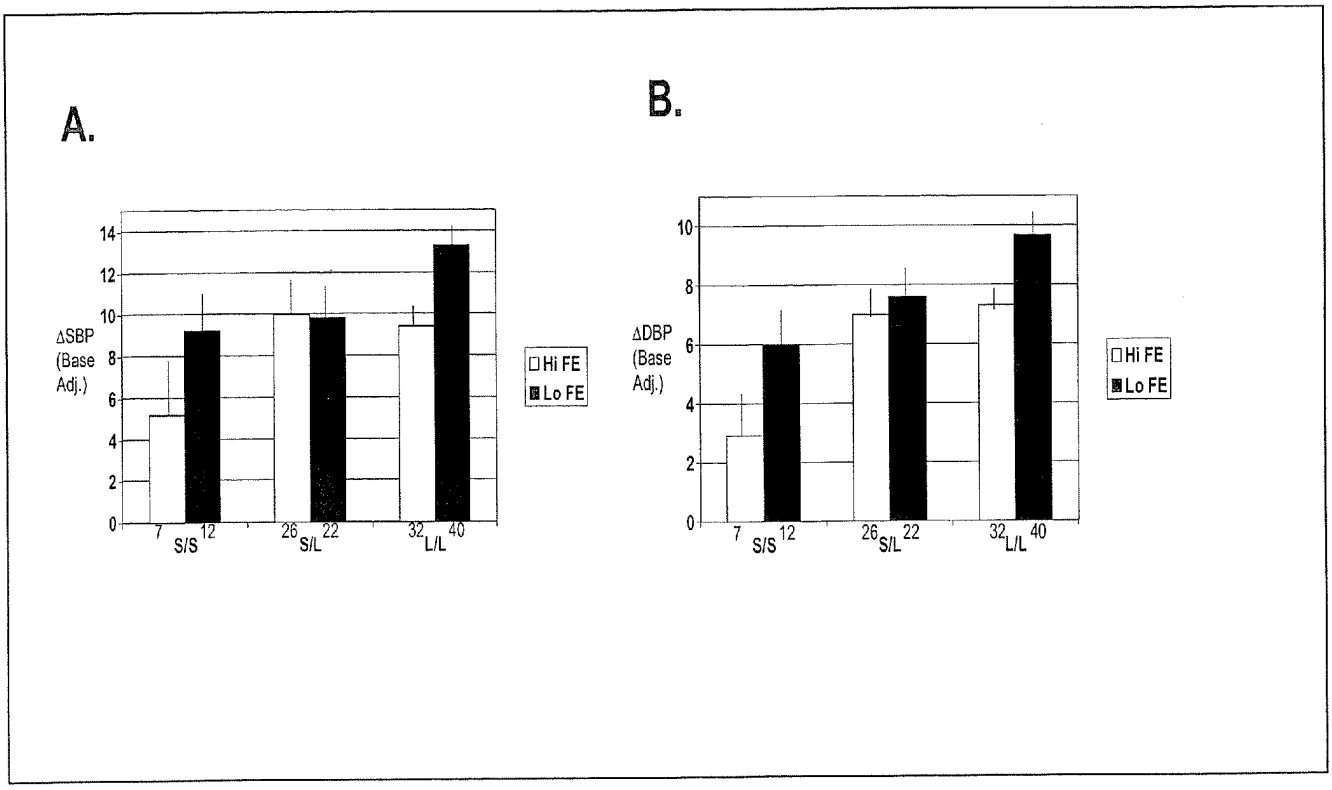
Father's Educ & 5HTTLPR Genotype (N)	SBP Change*	DBP Change	HR Change
Father's Educ			
Low (74)	10.8(0.8)	7.8(0.4)	8.2(0.6)
High (65)	8.2(1.0)	5.6(0.5)	6.9(0.7)
P	**0.041**	**0.002**	0.172
5HTTLPR			
LL(72)	11.4(0.7)[a]	8.6(0.4)[a]	8.7(0.6)[a]
LS (48)	9.9(0.9)	7.2(0.5)[b]	8.5(0.7)[a]
SS (19)	7.2(1.5)[b]	4.3(0.8)[c]	5.5(1.1)[b]
P	**0.047**	**<0.0001**	**0.033**

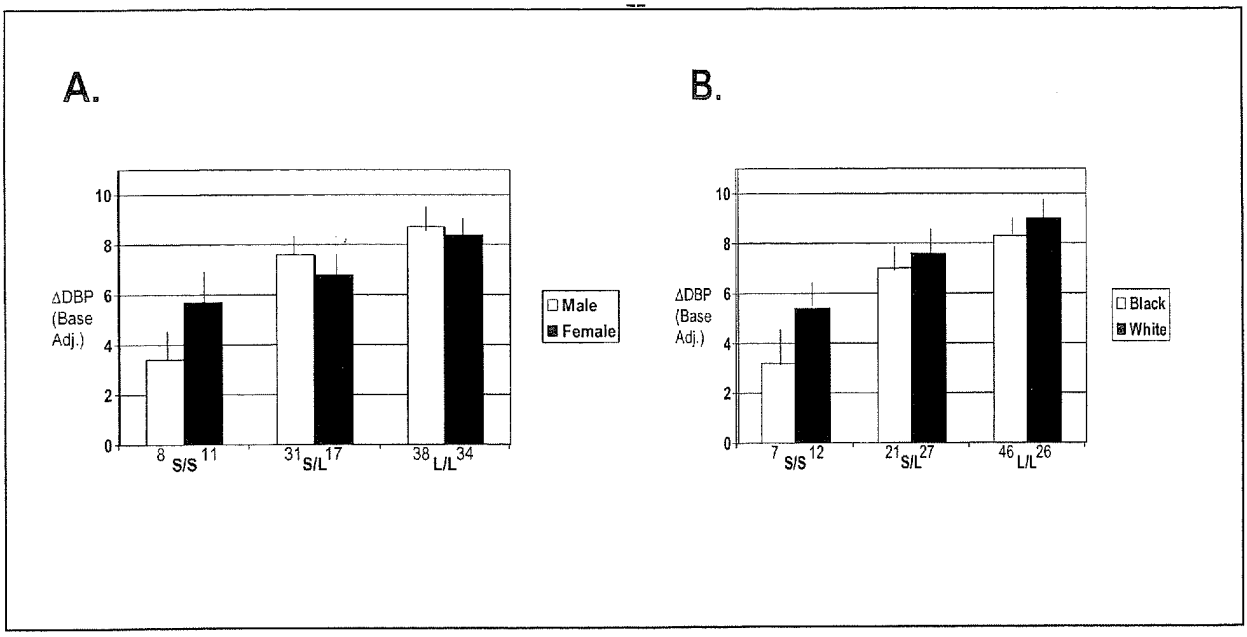
*Mean (S.E.)
[a]values with different letters for each CV measure are significantly different

Figure Legends

Figure 1. SBP (**A**) and DBP (**B**) responses (mean, S.E) to stress periods as a function of Father's Education level and 5HTTLPR genotypes. Race and baseline levels were entered first as covariates. (FE: Father's Education Level)

Figure 2. DBP responses (mean, S.E.) to stress periods as a function of 5HTTLPR genotypes and Sex (**A**) and Race (**B**). The effect of 5HTTLPR genotype on DBP reactivity was significant in blacks (P=0.004), whites (P=0.007) and males (P=0.002) and showed a trend (P=0.087) in females.


A.
ΔSBP (Base Adj.)
14
12
10
8
6
4
2
0
7
12
26
22
32
40
S/S
S/L
L/L
Hi FE
Lo FE
B.
ΔDBP (Base Adj.)
10
8
6
4
2
0
7
12
26
22
32
40
S/S
S/L
L/L
Hi FE
Lo FE


A.
ΔDBP (Base Adj.)
10
8
6
4
2
0
8 S/S 11
31 S/L 17
38 L/L 34
Male
Female
B.
ΔDBP (Base Adj.)
10
8
6
4
2
0
7 S/S 12
21 S/L 27
46 L/L 26
Black
White